JOURNAL OF CLINICAL MICROBIOLOGY, May 2000, p. 1823–1826 Vol. 38, No. 5
0095-1137/00/$04.00+0

Standardization of Immunoglobulin M Capture Enzyme-Linked Immunosorbent Assays for Routine Diagnosis of Arboviral Infections

DENISE A. MARTIN,* DAVID A. MUTH, TERESA BROWN, ALISON J. JOHNSON, NICK KARABATSOS, AND JOHN T. ROEHRIG

Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Public Health Service, U.S. Department of Health and Human Services, Fort Collins, Colorado 80522

Received 3 August 1999/Returned for modification 1 November 1999/Accepted 8 March 2000

Immunoglobulin M antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) is a rapid and versatile diagnostic method that readily permits the combination of multiple assays. Test consolidation is especially important for arthropod-borne viruses (arboviruses) which belong to at least three virus families: the *Togaviridae*, *Flaviviridae*, and *Bunyaviridae*. Using prototype viruses from each of these families and a panel of well-characterized human sera, we have evaluated and standardized a combined MAC-ELISA capable of identifying virus infections caused by members of each virus family. Furthermore, by grouping antigens geographically and utilizing known serological cross-reactivities, we have reduced the number of antigens necessary for testing, while maintaining adequate detection sensitivity. We have determined that a 1:400 serum dilution is most appropriate for screening antiviral antibody, using a positive-to-negative ratio of ≥2.0 as a positive cutoff value. With a blind-coded human serum panel, this combined MAC-ELISA was shown to have test sensitivity and specificity that correlated well with those of other serological techniques.

Most medically important arthropod-borne viruses (arboviruses) belong to three virus families: the *Togaviridae*, *Flaviviridae*, and *Bunyaviridae*. Many of these viruses are considered emerging or reemerging infectious diseases that can be readily transported from one area of the world to another. Because of the wide variety of viruses, arboviral diagnostic serology is complex. A recent survey of diagnostic laboratories in the United States that perform arboviral testing determined that the indirect immunofluorescence assay (2), plaque-reduction neutralization test (PRNT) (2, 17), hemagglutination-inhibition test (10), and complement fixation test (8) were still widely used (M. Bunning, personal communication). Many of these tests are technically demanding, making them difficult to apply reproducibly, and are often poor measures of the early antibody immunoglobulin M (IgM). Furthermore, obtaining results from these tests may take several days and require paired serum samples or live cell culture. Virus isolation is rarely a viable option even in epidemic situations, due to poorly timed specimens.

The IgM antibody-capture enzyme-linked immunosorbent assay (MAC-ELISA) was designed specifically to detect IgM antibody, which is a valuable tool for rapid diagnosis of acute viral infections. IgM appears early in infection, rises rapidly in the disease course, and is usually less virus cross-reactive than IgG (16). While many separate IgM ELISAs have been developed for arboviruses, these tests are not well standardized (1, 13, 15, 20). Most use a commercial source of anti-human IgM as capture antibody, but they also use purified virus as antigen, which is impractical for multiple agents (4–7, 11, 14). Furthermore, the selection of the screening serum dilution has not been extensively evaluated (7, 19) but has relied on results from an earlier study (21). These assays use polyclonal antiviral antibody as a detector, which is impractical for all but the largest reference laboratories that prepare these specialized reagents routinely (4–7, 11, 14). These polyclonal antibody detectors vary in batch-to-batch potency and can be quite virus cross-reactive, which limits test specificity. Finally, the criteria that indicate positive reactions, usually expressed as a positive-to-negative (P/N) ratio, have been inconsistent.

We have developed, implemented, and validated a standardized MAC-ELISA for rapid screening of human serum samples for various arboviruses. These new tests use a defined set of antigens, tailored to the geographic origin of the specimen. The method is readily standardizable, using a commercial source of anti-human IgM capture antibody and broadly reactive antiviral monoclonal antibody (MAb)-enzyme conjugates as detectors. This approach has resulted in a reliable, rapid, and readily transferable system for monitoring arbovirus disease.

MATERIALS AND METHODS

Human serum. Serum specimens were obtained from the Centers for Disease Control and Prevention, Division of Vector-Borne Infectious Diseases (DVBID), serum bank, which consists of specimens sent to DVBID for arboviral diagnostic testing. Sera were selected on the basis of a positive result to either eastern equine encephalitis virus (EEE), St. Louis encephalitis virus (SLE), or La Crosse encephalitis virus (LAC) in a previously performed serologic test. For the MAC-ELISA standardization, 22 positive and 13 negative sera were used for EEE, 24 positive and 12 negative sera were used for SLE, and 18 positive and 9 negative sera were used for LAC. Positive serum specimens with high, medium, and low reactivities were chosen.

PRNT. The serum dilution PRNT was performed with Vero cells, as previously described (2). The following viruses were used to represent the three viral genera in all tests: EEE strain NJ/60, SLE strain TBH-28, and LAC strain Original. Endpoints were determined at a 90% plaque-reduction level.

MAC-ELISA. This test was a modification of the assay previously reported by Beaty et al. (2). Goat anti-human IgM (PerImmune, Inc., Rockville, Md.) was used as capture antibody, and aliquots were stored at −70°C long-term, thawed once, and held at 4°C thereafter. Carbonate-bicarbonate buffer (0.015 M sodium carbonate, 0.035 M sodium bicarbonate, pH 9.6) was used as coating buffer.

* Corresponding author. Mailing address: Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, P.O. Box 2087, Fort Collins, CO 80522. Phone: (970) 221-6445. Fax: (970) 221-6476. E-mail: DZM9@CDC.GOV.

TABLE 1. Testing panels

Test panel	Arboviruses included[a]
Western United States	WEE, SLE, LAC, CE,[b] VEE,[c] DEN,[c] WN
Eastern United States	EEE, SLE, LAC, DEN,[d] EVE,[d,e] POW,[f] WN
Europe	SIN, TAH, POW/TBE,[g] WN
Central America and Caribbean	WEE, EEE, VEE, MAY, SLE, DEN
South America	WEE, EEE, VEE, MAY, SLE, YF, DEN
Africa	CHIK, SIN, YF, DEN, TAH, WN
Australia and Oceania	RR, BF, SIN, DEN, MVE
Asia and Middle East	CHIK, SIN, JE, POW/TBE, DEN, SSH, WN

[a] Virus abbreviations: WEE, western equine encephalitis; CE, California encephalitis; VEE, Venezuelan equine encephalitis; DEN, dengue, EVE, Everglades; POW, Powassan; SIN, Sindbis; TAH, Tahyna; TBE, tick-borne encephalitis; MAY, Mayaro; YF, yellow fever; CHIK, Chikungunya; RR, Ross River; BF, Barhmah Forest; MVE, Murray Valley encephalitis; JE, Japanese encephalitis; SSH, snowshoe hare, West Nile.

[b] California only.

[c] Texas only.

[d] Florida only.

[e] Subtype of Venezuelan equine encephalitis virus.

[f] Only along the eastern coast of the United States.

[g] Powassan virus and tick-borne encephalitis virus are closely related and cross-reactive. Tick-borne encephalitis virus antigen is not available in the United States; therefore, Powassan virus antigen is substituted.

Coated plates (Immulon II 96-well microtiter plates; Dynatech Industries, Inc.) were incubated overnight at 4°C in a humidified container and were stable for 2 weeks. A blocking step using phosphate-buffered saline with 0.5% Tween 20 and 5% nonfat dry milk was added to reduce overall background and increase test sensitivity. Blocking and coating buffers and undiluted conjugates after reconstitution were stored at 4°C. A standard five washes with a Skatron microplate washer (Skatron Instruments, Sterling, Va.) were used except before the addition of substrate where 10 washes reduced overall background. Sera and homologous positive and negative antibody controls were titrated using 10 twofold dilutions starting at 1:100 to evaluate the optimum screening dilution. The diluent used for test sera and antigens was phosphate-buffered saline with 0.5% Tween 20 without fetal bovine serum as recommended by Beaty et al. (2). Diluted antibody was stable for only 7 to 10 days and was discarded thereafter (personal observation). Viral and normal antigens, prepared as sucrose-acetone extracts of infected suckling mouse brains (2), were obtained from the DVBID reference collection. Reconstituted undiluted antigen was stored at −20°C, and antigen dilutions were used once and discarded. An overnight incubation at 4°C was used for the antigen step to increase test sensitivity, after observing a decrease in measured absorbance (A_{450}) values when using a 2-h antigen incubation. Group-reactive MAbs were purified and conjugated to horseradish peroxidase by Jackson Immunological Laboratories, Inc. (West Grove, Pa.), and used as detector antibodies. The following MAbs were used: 2A2C-3 for alphaviruses (15), 10G5.4 for California group viruses (a gift from George Ludwig via Barbara Israel, University of Wisconsin) (18), and 6B6C-1 for flaviviruses (22). Use of MAb detectors eliminated the necessity for a secondary antibody addition. Commercially prepared 3,3′,5,5′-tetramethylbenzidine (TMB-ELISA; GIBCO Bethesda Research Laboratories, Inc., Gaithersburg, Md.) was used, and the substrate reaction was stopped with 1 N sulfuric acid. Reactions were measured using a Bio-Rad microplate reader (Bio-Rad Laboratories, Hercules, Calif.) at an absorbance of 450 nm.

All reagents were titrated individually by using a twofold dilution series that provided reagent excess in the first well of the series. Reagent dilutions which resulted in optical densities between 0.8 and 1.0 were chosen.

Calculation of P/N values. Calculations that were performed followed guidelines set in the work of Beaty et al. (2) with the following modifications. The negative serum control P/N must be <2.0 and the positive serum control P/N must be ≥ 2.0 for the test to be valid. A positive test result was obtained when the P/N of the test serum was >2.0 and the mean of the A_{450} values of the test serum reacted on viral antigen was at least twice the mean of the A_{450} values of serum reacted on normal mouse brain antigen. When the latter criterion was not met due to nonspecific reaction with the normal mouse brain antigen, the result was reported as uninterpretable.

Validation testing panels. Seven positive sera from each virus group and seven negative sera (confirmed by MAC-ELISA and PRNT results) were selected, blind coded, and tested in triplicate by MAC-ELISA by using the 1:400 serum screening dilution. For viruses in the antigen batteries other than EEE, SLE, and LAC, at least three positive serum specimens were collected and analyzed using the screening MAC-ELISA.

RESULTS

Determination of antigen panels. We performed a historical analysis of arboviral testing results produced in our laboratory over the past 20 years. The vast majority of positive serum samples were representative of a few viruses that could be localized geographically. Although it has been shown previously that IgM is less cross-reactive than IgG (especially for alphaviruses) (5, 23), enough cross-reactivity exists especially in the family *Flaviviridae* (3, 19) and the California serogroup viruses (1, 7) to permit the establishment of seven geographically based testing panels representing the medically important arboviruses. These panels are shown in Table 1. Using these batteries to screen specimens ensured the detection of virtually all the endemic arboviruses in that area of the world for those virus families, which reduced the amount of testing needed for diagnosis, saving time and resources.

Determination of the MAC-ELISA screening dilution. Titration curves for the representative antiviral serum samples are shown in Fig. 1. The resultant curves were typical of ELISA endpoint titrations, which correlated well with concurrently run PRNT for the same sera. Figure 1 demonstrates the flat line produced by negative serum specimens even though in some cases the A_{450} values were sufficient for negative P/N values to be in the range of 2.0 to 2.5. To determine the optimum dilution for screening sera, P/N values for these sera

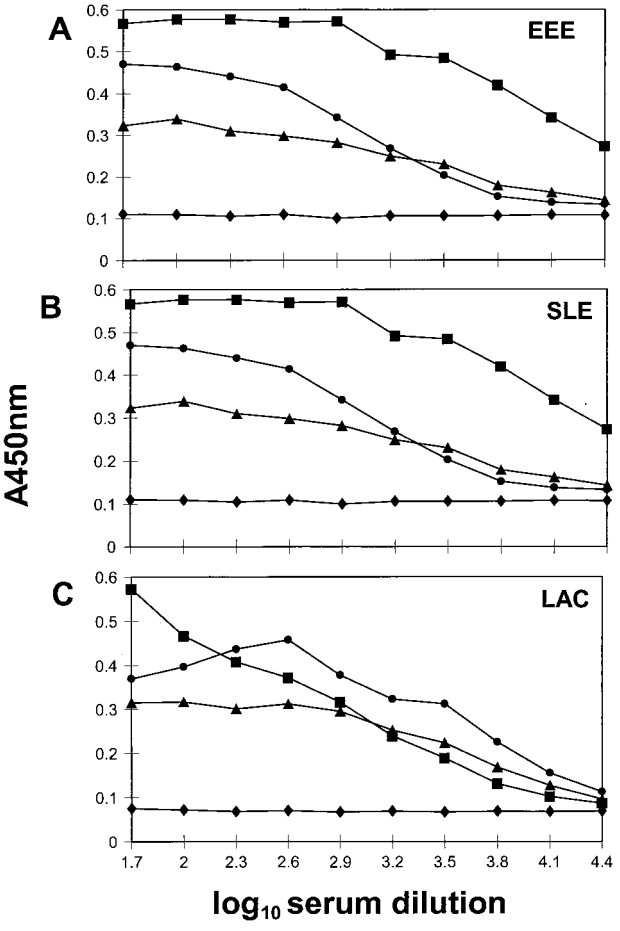

FIG. 1. MAC-ELISA titration curves. Log_{10} serum dilution versus A_{450} is presented. Representative specimens of high-titered sera (■), medium-titered sera (●), low-titered sera (▲), and negative sera (◆) are plotted.

TABLE 2. Results of coded sera tested by MAC-ELISA for all virus families

Serum no.	Antigen[a]	P/N ratio for virus[b]:		
		SLE	EEE	LAC
1	NEG	1.91	1.77	1.32
2	SLE	**5.09**	1.11	0.74
3	EEE	0.95	**6.94**	1.26
4	EEE	1.55	**3.12**	0.96
5	LAC	*2.07*	1.30	**3.60**
6	SLE	**6.43**	1.08	1.10
7	NEG	1.00	1.83	1.39
8	EEE	1.70	**12.53**	0.64
9	EEE	1.15	**4.15**	0.98
10	SLE	**7.03**	1.22	1.44
11	NEG	1.22	1.08	1.06
12	LAC	1.94	—	**6.97**
13	LAC	1.16	1.17	**4.01**
14	SLE	**4.46**	1.06	1.25
15	EEE	1.23	**7.91**	1.31
16	NEG	1.30	1.23	*2.73*
17	EEE	1.18	**5.36**	1.22
18	SLE	**7.35**	1.31	1.02
19	LAC	1.42	0.98	**6.39**
20	NEG	1.27	0.95	1.22
21	EEE	1.28	**8.43**	1.17
22	NEG	*2.60*	1.83	1.34
23	NEG	1.74	1.35	1.16
24	LAC	1.58	1.77	**8.37**
25	SLE	**2.59**	1.04	0.93
26	LAC	1.39	1.54	**6.75**
27	LAC	1.19	1.13	**4.10**
28	SLE	**11.30**	0.96	0.84

[a] Antigen for which serum tested positive in the original testing. NEG, negative.

[b] Boldface indicates a positive result for the correct virus; italics indicate a positive result for an incorrect virus. —, result uninterpretable because of high background.

at 1:100, 1:400, 1:1,600, and 1:25,600 dilutions were compared to endpoint titers. Using chi-square analysis for all three viruses tested, a 1:400 screening dilution generated P/N ratios in the positive range that correlated well with measured endpoint titers. Only 2 of 20 positive EEE antibody specimens were positive by endpoint but negative by 1:400 dilution screening. One of 12 positive LAC antibody specimens was positive by 1:400 dilution screening and negative by endpoint titration. The 22 specimens testing positive to SLE in the screening MAC-ELISA were also positive by endpoint titration. All antibody-negative control specimens were negative by both testing procedures for all three viruses.

Test validation. Results of the blind-coded serum testing are shown in Table 2. MAC-ELISA screening of sera testing positive to other members of the antigen batteries showed uniform positive reactions to homologous antigens. Little cross-reaction was observed within the alphaviruses; however, Chikungunya virus cross-reacted with o'nyong-nyong virus. Flaviviruses and California serogroup viruses exhibited extensive cross-reaction between members of each respective group.

DISCUSSION

An array of antibody types and subclasses is produced by a normal host humoral response to viral infection. IgM antibody is produced early in the immune response (16). The MAC-ELISA specifically detects IgM, allowing for timely diagnosis of disease. The capture format of the MAC-ELISA eliminates potential background caused by extraneous antibody, resulting in less-frequent nonspecific reactions and removing false-positive reactions caused by rheumatoid factor (12). Competition between IgM and IgG for antigen binding is minimized, reducing the occurrence of false-negative results. Ensuring exact incubation times, especially in the critical substrate step, yields results that can be compared between tests. The use of broadly group-reactive MAb conjugates in combination with virus-specific antigens creates a system in which antibody to many arboviruses within a genus can be screened for concurrently by using a single virus-adaptable procedure.

Correlation of the MAC-ELISA results with the PRNT was good. The occasional disparity that was noted between results of the two tests is probably explained by the fact that the antibody types detected can be different. IgM produced early in infection does not always possess neutralizing activity (7). When the worldwide antibody panels based on the various exploitable cross-reactions were used (1, 7, 17, 19), a single MAC-ELISA format incorporating various antigens gave a rapid and precise picture of the IgM antibody status of a given serum. Newly emerging arboviruses and those in other virus families can be added to any battery as soon as the proper viral antigens have been developed and positive controls are obtained. Using the MAC-ELISA in tandem with an IgG ELISA (14a) yields antibody profiles capable of identifying recent infections by separate measurement of IgM and IgG.

Interpretation of MAC-ELISA results is based primarily on the timing of the sample and confirmation of those results in another test. A potentially IgM-positive acute-phase serum sample is defined for purposes of this test as serum taken at least 8 days and up to 45 days after onset of symptoms (unpublished data). A positive result by MAC-ELISA in a single acute-phase specimen is presumptive evidence of a recent infection with that arbovirus (9), exploiting the early rising and rapidly declining nature of IgM antibody. The detection of IgM antibody to a particular arbovirus in a cerebrospinal fluid (CSF) specimen is evidence of infection with that virus (2, 9). However, a negative MAC-ELISA result for a very acute-phase specimen (day 0 to 7) may reflect an insufficient antibody response very early in infection rather than no infection. Moreover, MAC-ELISA results in an acute specimen that remain unconfirmed by PRNT may reflect the lack of neutralizing ability of IgM produced early in infection and should not be confused with a false-positive result (7). Routinely requesting a convalescent-phase specimen in this situation alleviates this dilemma. In the absence of a convalescent-phase specimen, PCR can be used for confirmation.

Our results showed that the screening dilution (1:400) had a good correlation with true endpoint values, therefore eliminating the need for endpoint titrations except for confirmation of positive serum detected by the initial screening MAC-ELISA. Occasionally, CSF specimens are submitted, and these are screened undiluted. Using a screening dilution of 1:400 eliminates most false positives. However, P/N values in the range of 2.0 to 3.0 have occasionally been determined to be false positives, requiring endpoint titrations to confirm the positive reaction (personal observation). In practice, we do MAC-ELISA endpoint titrations on serum specimens positive in our screening MAC-ELISA. False-positive sera yielding an endpoint generally graph as a flat line and demonstrate a fairly stable P/N range, usually under 3.0. However, only a portion of these sera with low P/N values will fail a confirmatory neutralization test.

Development of this adaptable and rapid MAC-ELISA for detection of medically important arboviruses in serum and CSF has made clinical diagnosis more efficient. During the West Nile encephalitis epidemic in New York City in the

summer of 1999, the MAC-ELISA was a key tool in early detection of cases. The use of SLE virus in the U.S. antigen panel allowed us to detect infection with WN in this new setting. This further demonstrated the versatility of virus panels, while making the process of arbovirus detection streamlined and more efficient. Nevertheless, the use of more traditional testing methods for some arboviruses that are not members of the families addressed here must continue. Use of the MAC-ELISA and IgG ELISA in tandem will allow us to examine the nature of antibody response in arboviral infections in more detail.

ACKNOWLEDGMENTS

We thank Alan Dupuis and Rebecca Deavours for their help with the preparation of the manuscript.

REFERENCES

1. **Artsob, H., L. P. Spence, and C. Th'ng.** 1984. Enzyme-linked immunosorbent assay typing of California serogroup viruses isolated in Canada. J. Clin. Microbiol. **20:**276–280.
2. **Beaty, B. J., C. H. Calisher, and R. E. Shope.** 1995. Arboviruses, p. 204–205. *In* E. H. Lennette, D. A. Lennette, and E. T. Lennette (ed.), Diagnostic procedures for viral, rickettsial and chlamydial infections, 7th ed. American Public Health Association, Washington, D.C.
3. **Calisher, C.** 1998. Antigenic classification and taxonomy of flaviviruses (family Flaviviridae) emphasizing a universal system for the taxonomy of viruses causing tick-borne encephalitis. Acta Virol. **32:**469–478.
4. **Calisher, C. H., O. Meurman, M. Brummer-Korvenkontio, P. E. Halonen, and D. J. Muth.** 1985. Sensitive enzyme immunoassay for detecting immunoglobulin M antibodies to Sindbis virus and further evidence that Pogosta disease is caused by a western equine encephalitis complex virus. J. Clin. Microbiol. **22:**566–571.
5. **Calisher, C. H., A. O. El-Kafrawi, M. I. Al-Deen Mahumud, A. P. A. Travassos da Rosa, C. R. Bartz, M. Brummer-Korvenkontio, S. Haksohusodo, and W. Suharyono.** 1986. Complex-specific immunoglobulin M antibody patterns in humans infected with alphaviruses. J. Clin. Microbiol. **23:**155–159.
6. **Calisher, C. H., V. P. Berardi, D. J. Muth, and E. E. Buff.** 1986. Specificity of immunoglobulin M and G antibody responses in humans infected with eastern and western equine encephalitis viruses: application to rapid serodiagnosis. J. Clin. Microbiol. **23:**369–372.
7. **Calisher, C. H., S. I. Pretzman, D. F. Muth, M. A. Parsons, and E. D. Peterson.** 1986. Serodiagnosis of La Crosse virus infections in humans by detection of immunoglobulin M class antibodies. J. Clin. Microbiol. **23:**667–671.
8. **Casey, H. L.** 1965. Standardized diagnostic complement fixation method and adaptation to microtest. Public health monograph no. 74. U.S. Government Printing Office, Washington, D.C.
9. **Centers for Disease Control and Prevention.** 1997. Case definitions for infectious conditions under public health surveillance. Morbid. Mortal. Weekly Rep. **46**(RR-10)**:**12–13.
10. **Clarke, D. H., and J. Casals.** 1958. Techniques for hemagglutination and hemagglutination-inhibition with arthropod-borne viruses. Am. J. Trop. Med. Hyg. **7:**561–573.
11. **Dittmar, D., T. Cleary, and A. Castro.** 1979. Immunoglobulin G- and M-specific enzyme-linked immunosorbent assay for detection of dengue antibodies. J. Clin. Microbiol. **9:**498–502.
12. **Feinstein, S., Y. Akov, B. Lachmi, S. Lehrer, L. Rannon, and D. Katz.** 1985. Determination of human IgG and IgM class antibodies to West Nile virus by enzyme linked immunosorbent assay (ELISA). J. Med. Virol. **17:**63–72.
13. **Frazier, C., and R. Shope.** 1979. Detection of antibodies to alphaviruses by enzyme-linked immunosorbent assay. J. Clin. Microbiol. **10:**583–585.
14. **Jamnback, T. L., B. J. Beaty, S. W. Hildreth, K. L. Brown, and C. B. Gundersen.** 1982. Capture immunoglobulin M system for rapid diagnosis of La Crosse (California encephalitis) virus infections. J. Clin. Microbiol. **16:** 577–580.

14a. **Johnson, A. J., D. A. Martin, N. Karabatsos, and J. T. Roehrig.** 2000. Detection of anti-arboviral immunoglobulin G by using a monoclonal antibody-based capture enzyme-linked immunosorbent assay. J. Clin. Microbiol. **38:**1827–1831.

15. **Karabatsos, N. K., A. L. Lewis, C. H. Calisher, A. R. Hunt, and J. T. Roehrig.** 1988. Identification of Highlands J virus from a Florida horse. Am. J. Trop. Med. Hyg. **39:**603–606.
16. **Kuby, J.** 1997. Immunology, 3rd ed., p. 399. W. H. Freeman and Co., New York, N.Y.
17. **Lindsey, H. S., C. H. Calisher, and J. H. Matthews.** 1976. Serum dilution neutralization test for California group virus identification and serology. J. Clin. Microbiol. **4:**503–510.
18. **Ludwig, G. V., B. A. Israel, B. M. Christensen, T. M. Yuill, and K. T. Schultz.** 1991. Monoclonal antibodies directed against the envelope glycoproteins of La Crosse virus. Microb. Pathog. **11:**411–421.
19. **Monath, T. P., R. R. Nystrom, R. E. Bailey, C. H. Calisher and D. J. Muth.** 1984. Immunoglobulin M antibody capture enzyme-linked immunosorbent assay for diagnosis of St. Louis encephalitis. J. Clin. Microbiol. **20:**784–790.
20. **Niklasson, B., C. J. Peters, M. Grandien, and O. Wood.** 1984. Detection of human immunoglobulin G and M antibodies to Rift Valley fever virus by enzyme-linked immunosorbent assay. J. Clin. Microbiol. **19:**225–229.
21. **Roehrig, J. T.** 1982. Development of an enzyme-linked immunosorbent assay for the identification of arthropod-borne togavirus antibodies. J. Gen. Virol. **63:**237–240.
22. **Roehrig, J. T., J. H. Mathews, and D. W. Trent.** 1983. Identification of epitopes on the E glycoprotein of Saint Louis encephalitis virus using monoclonal antibodies. Virology **128:**118–126.
23. **Westaway, E. G., A. J. Della-Porta, and B. M. Reedman.** 1974. Specificity of IgM and IgG antibodies after challenge with antigenically related togaviruses. J. Immunol. **112:**656–663.